Amendments to the Specification:

Please amend the specification by inserting the replacement paragraph below at page 1, lines 5-7.

The present application is a National Phase entry of PCT/KR03/00131, filed on January 21, 2003, which claims priority from ~~is based on~~ Korean patent application No. 2002-004297, filed on January 24, 2002, and Korean patent application No. 2002-0011648 filed on March 5, 2002.

Please amend the specification by inserting the replacement paragraph below at page 1, lines 12-15.

The present invention relates to a pair of primers specific to ~~Mycobacterial~~ mycobacterial species, more specifically to a pair of primers that can specifically amplify the hsp 65 gene of mycobacteria, a gene fragment of hsp 65, and an identifying method of ~~Mycobacterial~~ mycobacterial species.

Please amend the specification by inserting the replacement paragraph below at page 1, lines 12-15.

The genus ~~Mycobacterium~~ *Mycobacterium* covers a wide range of organisms including obligate species causing serious human and animal disease such as tuberculosis, bovine tuberculosis, and leprosy; opportunistic pathogens; and saprothytic species found in the natural environment. At present, it is known that about 72 species of the genus ~~mycobacterium~~ *Mycobacterium* have been reported, of which about 25 species are involved in the human diseases.

Please amend the specification by inserting the replacement paragraph below at page 2, lines 1-12.

Tuberculosis is the largest of the ~~Mycobacterial~~ mycobacterial infections. The ~~Mycobacterial~~ mycobacterial species causing tuberculosis include ~~M. tuberculosis, M bovis, M.~~

~~africanum, and M. microti~~ *M. tuberculosis, M bovis, M. africanum, and M. microti*, which are classified as ~~M. tuberculosis~~ *M. tuberculosis* complex (TB complex). M. tuberculosis is common and important in causing tuberculosis. Tuberculosis infection decreased because of continuous use of antituberculosis drugs until the end of the 1980s, but in line with the rapid increase of AIDS and Mycobacterium tuberculosis with drug resistance, tuberculosis increased in developed countries in the 1990s. In particular, it has been reported that the death rate due to tuberculosis is the highest among infectious diseases in Korea, claiming about three hundred or more lives per year, because of the increase in the number of street people in the International Monetary Fund era in Korea.

Please amend the specification by inserting the replacement paragraph below starting at page 2, line 13 through page 3, line 3.

Mycobacteria Other than ~~Mycobacterium tuberculosis~~ *Mycobacterium tuberculosis* (MOTT, or nontuberculous mycobacteria, NTM) causes infection in aged people and immuno-compromised patients, and its clinical manifestation is similar to tuberculosis. The occurrence of MOTT is still lower than tuberculosis in Korea, but it is quite common. It is difficult to determine the pathogenicity from isolate that is separated from a clinical sample. In addition, resistance of MOTT to most anti-tuberculosis drugs and its recurrence rate makes it difficult to treat MOTT infection. It has been reported that MOTT also ~~causes~~ cause disease in patients who are not immuno-compromised, and that 50% of ~~Mycobacterial~~ mycobacterial infection in the United States is tuberculosis and 50% is MOTT infection over the past 10 years. With the spread of HIV (Human immunodeficiency virus) infection since the 1980s, MOTT has caused systemic disseminated infection of immuno-compromised patients. Thus, MOTT has been closely watched.

Please amend the specification by inserting the replacement paragraph below at page 3, lines 4-8.

Mycobacterial species have different patterns of resistance to antituberculosis drugs from each other, and thus they are treated by different methods with different drugs (Wolinsky E:

Mycobacterial diseases other than tuberculosis. *Clin Infect Dis* 15: 1-10, 1992). Accordingly, ~~Mycobacteria~~ mycobacteria need to be differentiated and identified on a species level.

Please amend the specification by inserting the replacement paragraph below at page 3, lines 9-20.

A biochemical method for identifying ~~Mycobacterial~~ mycobacterial species is laborious and time-consuming due to the slow growing rate of ~~Mycobacteria~~ mycobacteria. A cell wall lipid analyzing method using High-performance Lipid Chromatography (HPLC) and Thin Layer Lipid Chromatography (TLC) is difficult to perform and is costly, and thus it is carried out on a small laboratory scale. The use of conventional identifying methods has a disadvantage in that it takes a great deal of time to perform due to the slow growing rate of the ~~Mycobacteria~~ mycobacteria (about 2-3 months for slow-growing mycobacteria). Thus, the treatment of ~~Mycobacterial~~ mycobacterial infection can be delayed (Nolte FS, Metchock B: *Mycobacterium*, In Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH (eds.), Manual of clinical microbiology. American Society for Microbiology, Washington, D.C. 400-437, 1995.).

Please amend the specification by inserting the replacement paragraph below beginning at page 3, line 21 through page 4, line 5.

16s rDNA is commonly used as a chronometer molecule for identification of the ~~Mycobacterial~~ mycobacterial species with a molecular biological method. In 1990, the nucleic acid sequence of 16s rDNA was analyzed, and it shows the phylogenetic relationship of ~~Mycobacteria~~ mycobacteria well. Until now, various methods of identifying ~~Mycobacterial~~ mycobacterial species by using the 16S rDNA have been developed and studied (Comparative sequence analysis, Probe hybridization, and Polymerization chain, reaction-restriction fragment length polymorphism).

Please amend the specification by inserting the replacement paragraph below beginning at page 3, line 6-22.

Identifying methods of ~~Mycobacterial~~ mycobacterial species by using dnaJ and 23S rDNA as alternative chronometers were developed in 1994. However, dnaJ and 23S rDNA have problems in phylogenetic relationship determination and conservation of nucleic acid sequences, and thus the methods were not used for target genes (Victor TC, Jordaan AM, Van Schalkwyk EJ, Coetzee GJ, Van Helden PD. Strain-specific variation in the dnaJ gene of mycobacteria. J Med Microbiol. 44(5):332-339, 1996). In 1993, Telenti A et al. reported that a method for the identification of mycobacteria at the species level was developed by using polymerase chain reaction (PCR)-Restriction Enzyme Length Polymorphism of a gene fragment of hsp 65. The method involves steps of amplifying an hsp 65 gene fragment by PCR and restriction enzyme analysis of PCR products of hsp 65 with two restriction enzymes, BstEII and HaeIII, and 29 species and subspecies were differentiated by PCR-restriction enzyme pattern analysis. (Telenti A, Marchesi F, Balz M, Bally F, Bottger EC, Bodmer T. "Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis," J. Clin. Microbiol. 31(2):175-8. 1993).

Please amend the specification by inserting the replacement paragraph below beginning at page 8, line 5-6.

Figs. ~~7a to 7d~~ 7A to 7D shows the results of the identification of mycobacteria in a clinical sample according to a comparative sequence analysis.

Please amend the specification by inserting the replacement paragraph below beginning at page 7, line 11-12.

Fig. 1 shows the hsp 65 gene fragment and the primers of the present invention, namely SEQ ID NO: 55 and SEQ ID NO: 56;

Please amend the specification by inserting the replacement paragraph below beginning at page 8, line 14-22.

Considering the problems in conventional identification methods and the taxonomy of mycobacteria, the inventors provide PCR primers that can amplify ~~M. tuberculosis~~ *M.*

tuberculosis and non-tuberculosis mycobacteria, an hsp 65 gene fragment as a chronometer molecule which exists in all mycobacteria, and a method for the identification of mycobacteria by using the primers and hsp 65 gene fragments. By using the restriction fragment of the amplified product of hsp 65 genes with treatment of Xho I, it is possible to differentiate ~~M. tuberculosis~~ *M. tuberculosis* and non-tuberculosis mycobacteria, and to differentiate non-tuberculosis mycobacteria.

Please amend the specification by inserting the replacement paragraph below beginning at page 10, lines 1-7.

The chronometer molecule used for the identification of mycobacterial species in the present invention is the 644-bp gene fragment located at the 163rd position to the 806th position of a 1623-bp hsp 65 gene of ~~M. tuberculosis~~ *M. tuberculosis*. The 644-bp gene fragment is substantially a 604-bp fragment because the 40-bp primer sequence is excluded. As a result of a Genbank database search, it was found that all 604-bp gene fragments of hsp 65 of 54 kinds of reference mycobacterial species are novel.

Please amend the specification by inserting the replacement paragraph below beginning at page 10, lines 8-16.

To establish the database for detecting and identifying the mycobacteria, the reference strains as shown in Table 1 were employed. 50 reference strains included 47 reference strains from the American Type Culture Collection (ATCC), a reference strain of ~~M. leprae~~ *M. leprae* (Thai 53 strains) from Hanssen's disease center of the Catholic University of Korea, and 2 reference strains (type II, III) of *M. kansasii* from V. Vincent. In addition, hsp 65 gene fragments of 3 reference strains of ~~Tsukamurella~~ *Tsukamurella* from the German Collection of Microorganisms and Cell Cultures, and a reference strain of *Nocardia* from ATCC were analyzed (Table 1).

Please amend the specification by inserting the replacement paragraph below beginning at page 12, line 1 - page 13, line 2.

For detecting and identifying ~~Mycobacterial~~ mycobacterial species, the present invention provides 604-bp hsp 65 gene fragments as a new chronometer molecule, instead of 16S rDNA. The chronometer molecules must satisfy the following requirements in order to reflect the phylogenetic relationship. Firstly, the target gene must be essential for the functions and be highly conserved in all organisms. Secondly, the target gene must not mutate by lateral transfer based on selection pressure between species. Thirdly, the target gene must have interspecies variation and intraspecies conservation, which suitably reflects a phylogenetic relationship. The hsp 65 gene fragment of the present invention suitably satisfies the requirements of the chronometer molecule.

Please amend the specification by inserting the replacement paragraph below beginning at page 14, line 8 - page 15, line 2.

The phylogenetic tree of the reference strains of the present invention showed the natural relationships of the mycobacteria. That is, the result confirmed that 50 reference strains of TB complex formed a large group excluding *T. paurometabola* as an outgroup (Fig. 6). Also, slow-growing mycobacteria and fast-growing mycobacteria formed different groups. *M. tuberculosis* and *M. leprae* of pathogenic mycobacteria formed the same branch of the phylogenetic tree. MOTT were isolated frequently. ~~M. avium~~ *M. avium* and ~~M. intracellulare~~ *M. intracellulare*, showing quite similar biochemical characteristics, formed the same branch. The results showed general characteristics of mycobacteria. ~~M. kansasii~~ *M. kansasii* and ~~M. gastri~~ *M. gastri* have 100% sequence homology, and thus cannot be differentiated according to the conventional identification method using 16S rDNA, but they are differentiated according to the present invention. Moreover, the subspecies of ~~M. kansasii~~ *M. kansasii* can be differentiated (namely, the hsp 65 gene fragments of *M. kansasii* Type I, II, and III have different nucleotide sequences).

The results of the present invention show the phylogenetic relationships of mycobacteria. That is, the slow-growing mycobacteria and fast-growing bacteria form different branches of the phylogenetic tree, and ~~M. tuberculosis~~ *M. tuberculosis* and ~~M. lepare~~ *M. lepare* form the same branch.

Please amend the specification by inserting the replacement paragraph below beginning at page 15, line 19 - page 16, line 1.

The general PRA method ~~comprising~~ comprises the steps of 1) DNA extraction, 2) PCR amplification, 3) confirmation of the amplified product, 4) digestion with a restriction enzyme, 5) analysis of restriction fragment, and 6) visualization by image capture systems. The restriction enzymes applicable for this invention include all the restriction enzymes that can recognize the site specifically existing in a 644-bp fragment, preferably Xho I.

Please amend the specification by inserting the replacement paragraph below beginning at page 17, lines 1-14.

The present invention provides a new system where a 644-bp hsp 65 gene fragment of mycobacteria is amplified with primers specifically for amplifying the 644-bp hsp 65 gene fragment of mycobacteria, and it is treated with Xho-I to differentiate and identify the mycobacterial species. Only a process of PRA makes it possible to differentiate the MOTT into 3 groups, as well as ~~M. tuberculosis~~ *M. tuberculosis*. That is, the treatment of the amplified product with a restriction enzyme produces only a 644-bp gene fragment in fast-growing mycobacteria, thereby differentiating it from the slow-growing mycobacteria. *M. avium* complex (for examples, *M. avium* and *M. intracellulare*) which belongs to slow-growing mycobacteria and is isolated most frequently in clinical samples produces three kinds of restriction fragments, 391-bp, 169-bp, and 84-bp, thereby differentiating them from other groups including *M. kansasii* producing two kinds of restriction fragments, 391-bp and 253-bp.

Please amend the specification by inserting the replacement paragraph below beginning at page 17, lines 15-19.

Among the genus ~~Mycobacterium~~ *Mycobacterium* that includes about 70 species, about 10 strains including *M. tuberculosis*, *M. avium* complex, *M. kansasii*, *M. szulgai*, *M. gordonae*, *M. fortuitum*, *and M. chelonae* cover 90% of isolates in a clinical sample, and thus they can be effectively identified according to the identification method of the present invention.

Please amend the specification by inserting the replacement paragraph below beginning at page 21, line 19 - page 22, line 6.

As the hsp 65 gene fragments of the mycobacterial species of interest are different from those of the reference species, mycobacterial species of interest can be identified based on the criterion of nucleotide sequence homology of hsp 65 genes of reference species. Because a mycobacterial species has a different range of sequence homology, mycobacterial species can be identified based on the specific range of the sequence homology thereof. For example, ~~M. gordonae~~ *M. gordonae* has a wide range of sequence homology, but ~~M. tuberculosis~~ *M. tuberculosis* has a narrow range. In addition, mycobacterial species can be identified by multi-aligning the nucleotide sequence of 604-bp hsp 65 gene fragments with those of reference species to infer a phylogenetic relationship.

Please amend the specification by inserting the replacement paragraph below beginning at page 25, lines 1-7.

The nucleotide sequences of 38 mycobacteria obtained from the clinical sample were analyzed and then multi-aligned with the database of reference strains to infer the phylogenetic tree. From the results, all 38 strains were identified to the species level with 100% sensitivity and specificity (Table 2 and Figs. [[7a]] 7A to [[7d]] 7D). The results are specifically described below.

Please amend the specification by inserting the replacement paragraph below beginning at page 25, line 9 - page 26, line 11.

~~M. tuberculosis~~ *M. tuberculosis* is the most pathogenic and important species in public health. The results of the identification of M. tuberculosis by using the database of the reference species of mycobacteria of the present invention confirmed that all twenty (20) ~~M. tuberculosis~~ *M. tuberculosis* were identified (Table 2 and Fig. 7c), and showed that 604-bp hsp 65 gene fragments of 20 strains have 100% sequence homology with a 604-bp fragment of ~~M. tuberculosis~~ *M. tuberculosis* ATCC 27284 reference strain. The 16s rDNA and rpoB gene used as a target gene are involved in resistance to streptomycin and to rifampin, respectively. The target genes in mycobacteria with a resistance to antituberculosis drugs can be mutated. However, unlike 16s rDNA and rpoB, the hsp 65 gene is not related to resistance to antibiotics, and thus it does not mutate. Therefore, the 604-bp hsp 65 gene is stable with respect to the selection pressure of antituberculosis drugs in comparison with other target genes.

Please amend the specification by inserting the replacement paragraph below beginning at page 26, line 13 - page 27, line 6.

The identification method was applied to 4 strains of ~~M. avium~~ *M. avium* complex which are the most commonly isolated in MOTT. As a result, the strains were identified to the species level as 3 ~~M. intracellulares~~ *M. intracellulares* and a strain of ~~M. avium~~ *M. avium*. According to the biochemical identification method, it is not possible to differentiate ~~M. intracellulare~~ *M. intracellulare* and ~~M. avium~~ *M. avium* because they have the same biochemical characteristics. In comparing the nucleotide sequences of 1 ~~M. avium~~ *M. avium* (KIT 41110) and ~~M. avium~~ *M. avium* ATCC 25281, they have 99.5% nucleotide sequence homology with 3 different nucleotides. When the nucleotide sequences of the 3 ~~M. intracellulares~~ *M. intracellulares* (KIT 41105, 41111, and 51115) are compared with that of ~~M. intracellulare~~ *M. intracellulare* ATCC 13850, they show 99.0-99.8% sequence homology. Those results are consistent with the fact that

~~M. intracellulare~~ *M. intracellulare* includes various genotypes, namely interspecies heterogeneity (Devallois A, Picardeau M, Paramasivan CN, Vincent V, Rastogi N: Molecular characterization of *Mycobacterium avium* complex isolates giving discordant results in AccuProbe tests by PCR-restriction enzyme analysis, 16s rRNA sequencing, and DT1-DT6 PCR. *J Clin Microbiol* 1997 **35:** 2767-2772).

Please amend the specification by inserting the replacement paragraph below beginning at page 27, lines 8-10.

2 strains (KIT 30101, 30102) were identified as *M. scrofulaceum* (Fig. [[7b]] 7B), and they have 99.8-100% nucleotide sequence homology with *M. scrofulaceum* ATCC 19981.

Please amend the specification by inserting the replacement paragraph below beginning at page 27, line 12 - page 28, line 2.

M. kansasii is the most pathogenic in MOTT, and it is in second position in isolation frequency after ~~M. avium~~ *M. avium* complex. 16s rDNA of ~~M. kansasii~~ *M. kansasii* showed 100% nucleotide sequence homology with that of nonpathogenic ~~M. gastri~~ *M. gastri*, thereby making it difficult to differentiate them. In addition, *M. kansasii* consists of at least 5 subspecies where type II and type III are reported to be separated from clinical material. 3 strains are identified as *M. kansasii* by using the database of 604-bp hsp 65 gene fragments of reference strains, which are consistent with results of the biochemical identification method. The method for identifying the mycobacterial species by using the database has characteristics such that *M. kansasii* can be differentiated from *M. gastri*, and subspecies of *M. kansasii* can be differentiated. The result confirmed that 3 strains of *M. kansasii* (KIT 20118, 20119, 20120) have 100% nucleotide sequence homology, and they are identified as *M. kansasii* Type I ATCC 12478 (Fig. 4c).

Please amend the specification by inserting the replacement paragraph below beginning at page 28, lines 3-18.

E. Identification of ~~M. gordonae~~ *M. gordonae*, ~~M. szulgai~~ *M. szulgai*, ~~M. marinum~~ *M. marinum*, and ~~M. terrae~~ *M. terrae* complex

As a result of identifying the clinically separated mycobacterial strains with the database of the present invention, 4 strains (KIT 32101, 32104, 32105, and 32106) were found to be *M. gordonae* (Fig. [[7a]] 7A, and Table 2). When comparing the nucleotide sequences of the 604-bp hsp 65 gene fragments of the 4 strains, they have 99.2-99.8% sequence homology with each other, but they have 95.9-96.3% sequence homology with *M. gordonae* ATCC 14470, which indicates a considerably low sequence homology between *M. gordonae* species. The result is consistent with the report that *M. gordonae* has intraspecies heterogeneity (Abed Y, Bollet C, de Micco P. Identification and strain differentiation of Mycobacterium species on the basis of DNA 16S-23S spacer region polymorphism. Res Microbiol. 1995 146(5): 405-13). That is, 4 isolates obtained from the same region have high sequence homology with one another, but low sequence homology with reference strains obtained from different regions.

Please amend the specification by inserting the replacement paragraph below beginning at page 28, line 19 - page 29, line 1.

As a result of identification of mycobacterial species with the database of reference species, 4 strains (KIT 31102, 31103, 31106, and 31107) were identified as *M. szulgai*, which is consistent with that of the biochemical identification method (Fig. [[7a]] 7A, and Table 2). The nucleotide sequences of the 4 strains have 99.5-100% nucleotide sequence homology with *M. szulgai* ATCC 35799.

Please amend the specification by inserting the replacement paragraph below beginning at page 29, lines 2-6.

As a result of identification of mycobacterial species with the database of reference species, 1 strain was identified as *M. marinum*, which is consistent with that of the biochemical identification method (Fig. [[7a]] 7A and Table 2). The nucleotide sequence of the strain has 99.3% nucleotide sequence homology with *M. marinum* ATCC 927(//).

Please amend the specification by inserting the replacement paragraph below beginning at page 30, line 21 - page 31, line 5.

As shown in Table 1, The hsp 65 gene fragments of 50 reference strains were sequenced, including 47 reference strains from the American Type Culture Collection (ATCC), a reference strain of ~~M. leprae~~ *M. leprae* (Thai 53 strain) from Hanssen's disease center of the Catholic university of Korea, and 2 reference strains (type II, III) of *M. kansasii* from V. Vincent. In addition, 3 reference strains of Tsukamurella from the German Collection of Microorganisms and Cell Cultures and a reference strain of *Nocardia* from ATCC were selected.